Preliminary Investigation of the C-terminal Mutations that cause Malignant Hyperthermia

A thesis presented to Massey University in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry

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2000

ACKNOWLEDGMENTS

I especially would like to thank my supervisor Dr. Kathryn Stowell for all her time, enthusiasm, expertise and support during this research project. I would also like to thank everyone else in the Twilight Zone, especially Carole Flyger, for their helpfulness. I also acknowledge the support of the Massey Masterate Scholarship which I was awarded during one of my years here. And I better say thanks to Mum and Dad who now know where ryanodine receptors are!

ABSTRACT

Malignant hyperthermia (MH) is a genetic disorder characterised by abnormal muscle contractures, hypermetabolism and hyperthermia. It is referred to as 'malignant' as it can lead to death when under anaesthetic if not recognised and treated immediately. The molecular basis of MH is an abnormality in the calcium release mechanism of the sarcoplasmic reticulum. Abnormal calcium release causes the physiological symptoms of an MH crisis. Genetic linkage studies have lead to the identification of the ryanodine receptor/Ca^{2+}-release channel as a causative factor in MH. The ryanodine receptor is a large protein which is regulated by a number of ligands including Ca^{2+}, Mg^{2+}, ATP, ryanodine and calmodulin.

Most mutations in the ryanodine receptor gene that cause MH are located near two main regulatory regions on the receptor. Three mutations have recently been identified that are located in the regulatory region of the C-terminal domain. The biochemical properties of one of these mutations have been studied. The current research project began to investigate the biochemical characteristics of the other two mutations in the C-terminal domain in relation to their ryanodine binding and calcium release properties.

Sarcoplasmic reticulum vesicles were isolated from skeletal muscle samples, and an attempt to identify ryanodine receptors by ^{3}H-ryanodine binding was made. RT-PCR using RNA extracted from a skeletal muscle sample was used to construct the cDNA for the C-terminal transmembrane domain of the ryanodine receptor. This cDNA was cloned into a mammalian expression vector and introduced into COS cells. RT-PCR was also used to produce the cDNA encoding a small polypeptide to an antigenic region in the C-terminal domain of the ryanodine receptor for the preparation of antibodies.

Although it appeared that there may have been ryanodine receptors in the SR vesicle preparation as determined by immunoblotting, ^{3}H-Ry binding to the ryanodine receptors was unable to confirm the presence of the receptors in the SR vesicles. Initial expression studies of the C-terminal domain in COS cells were inconclusive. Partial cleavage of a small antigenic polypeptide was obtained which could be used to produce antibodies to the C-terminal domain of the ryanodine receptor.

ABBREVIATIONS

^{3}H-Ry	tritiated ryanodine
amp	ampicillin
AMP-PCP	adenyl-(β,γ-methylene)-diphosphonate tetrathium salt
AMV	avian myeloblastosis virus
bp	base pair
BSA	bovine serum albumin
CaM	calmodulin
CHAPS	3-(3-cholamido-propyl-dimethylamino)-1-propanesulfonate
CHO	Chinese hamster ovary cells
C-terminal	carboxy terminal
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
DEPC	diethylpyrocarbonate
DHPR	dihydropyridine receptor
DMSO	dimethyl sulfoxide
DNase I	deoxyribonuclease one
dNTPs	dinucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
FCS	fetal calf serum
FKBP12	FK506 binding protein 12 kDa
GCG	genetics computing group
GuHCl	guanidium hydrochloride
GST	glutathione S-transferase
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulfuric acid
HRP	horse radish peroxidase
IPTG	isopropyl-β-D-thiogalactoside
kan	kanamycin

kb	kilobase
kDa	kilo Dalton
LB	luria broth
MEM	minimum essential medium
Mes	4-morpholino ethane sulfonic acid
MH	malignant hyperthermia
MHS	malignant hyperthermia susceptible
M-MLV	Moloney mouse leukaemia virus
MOPS	4-morpholine propane sulfonic acid
MWM	molecular weight marker
N-terminal	amino terminal
oligo	oligonucleotide
oligo(dT)	oligodeoxythymidine
PAGE	polyacrylamide gel electrophoresis
Pipes	1,4-piperazinediethane sulfonic acid
Pfu	*Pyrococcus furiosus*
RNase	ribonuclease
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
RyR1	skeletal muscle ryanodine receptor
RYR1	skeletal muscle ryanodine receptor gene
RyR-C	ryanodine receptor antibodies to the C-terminal domain
RyR-N	ryanodine receptor antibodies to the N-terminal domain
SDS	sodium dodecyl sulfate
spec	spectinomycin
SR	sarcoplasmic reticulum
Taq	*Thermus aquaticus*
TCA	trichloroacetic acid
TEMED	N,N,N′,N′-tetramethylethylenediamine
T_m	melting temperature
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet light

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CHAPTER ONE – INTRODUCTION

1.1 Malignant Hyperthermia

1.1.1 Introduction

Malignant Hyperthermia (MH) is a dominantly inherited, autosomal disorder of skeletal muscle, which is primarily triggered by exposure to volatile anaesthetic agents or depolarising muscle relaxants. This can lead to a sudden hyperpyrexia due to hypermetabolism and muscle breakdown. If not recognised and treated it progresses to a worsening hyperpyrexic state, muscle contractures, muscle rigidity, cardiac arrhythmias and other systemic effects or death (MacLennan and Phillips, 1992).

MH is considered the most common cause of anaesthetic death in otherwise fit and healthy people. However, with early recognition and intervention with the correct treatment, the fatality rate has decreased from ~70% to ~5% (Denborough, 1998).

1.1.2 Genetics of Malignant Hyperthermia

Extensive genetic analysis has lead to the identification of 25 point mutations in the skeletal muscle ryanodine receptor (*RYR1*) gene. In biochemical and physiological studies at least 21 of them have been shown to have a causal role in MH (Tong *et al.*, 1997; Jurkat-Rott *et al.*, 2000; McCarthy *et al.*, 2000). A mutation in a second gene encoding the α1-subunit of the dihydropyridine receptor (Monnier *et al.*, 1997) has been shown to be responsible for MH in some families. These mutations account for only ~50% of MH cases (McCarthy *et al.*, 2000; Richter *et al.*, 1997).

While the main MH locus is found on chromosome 19q13.1 and encodes the *RYR1* gene, genetic linkage studies have indicated the location of several other loci. Other genes that may be involved in MH susceptibility have been mapped to chromosome 1q31-q32,

3q13.1, 5p, 7q21-22, 7q21.1, 17q and 17q11.2-q24 (reviewed in Jurkat-Rott *et al.*, 2000; Loke and MacLennan, 1998; Mickelson and Louis, 1996).

1.1.3 Biochemistry of Malignant Hyperthermia

Initially, skeletal muscle was implicated in MH after observation of muscle contractures and an increase in serum creatine kinase levels after an MH reaction (Denborough *et al.*, 1970a; Denborough *et al.*, 1970b). Creatine kinase is a protein released from muscle cells, which accumulates in the blood and is an indicator of the level of damage that has occurred to muscle cells.

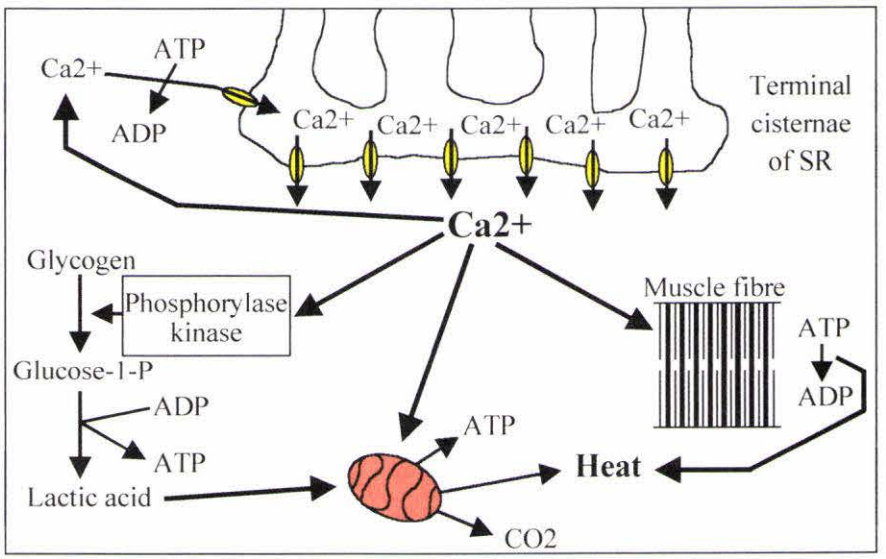

Figure 1.1: The molecular effects of calcium on muscle contraction and metabolism.

An increase in myoplasmic free calcium levels in skeletal muscle cells causes prolonged muscle contraction and enhanced metabolic processes. These actions lead to the biochemical events that manifest as Malignant Hyperthermia (Adapted from MacLennan and Phillips, 1992).

Indeed, the main molecular defect in MH appears to be an abnormality in the Ca^{2+}-release mechanism in the sarcoplasmic reticulum (SR) of skeletal muscle (Mickelson *et al.*, 1986). This causes a sustained release of calcium from the SR causing an increase in the myoplasmic calcium concentration and it is this calcium level that regulates muscle contraction and metabolic activity. In normal muscle cells calcium is released from the SR via Ca^{2+}- release channels and pumped back in via Ca^{2+}-ATPases. The released calcium is used to initiate muscle contraction by binding to troponin in the thin filaments, and activating glycolysis and aerobic metabolism by binding to and activating phosphorylase kinase. In MH muscle, calcium release is increased and consequently muscle contraction and metabolism is enhanced which accounts for the symptoms of MH (Figure 1.1).

The Ca^{2+}-release channel provides the only cellular site for the binding of the plant alkaloid ryanodine (Phillips *et al.*, 1996) and hence allows for the isolation, purification and biochemical study of the channel (Inui *et al.*, 1987). The properties of ryanodine binding to the Ca^{2+}-release channel led to it becoming known as the ryanodine receptor (RyR1).

Ryanodine binding is an indirect means of measuring calcium release as it binds to the receptor with high affinity, in a calcium-dependent manner, when in the open state (Mickelson and Louis, 1996; Valdivia *et al.*, 1991). Specific ligands that modulate calcium release also affect ryanodine binding in the same way. Therefore ryanodine binding reflects the functional state of the channel in that channel activators or inhibitors have an enhancing or suppressing effect on ryanodine binding (Palnitkar *et al.*, 1997; Richter *et al.*, 1997).

Calcium release can be modulated by a number of physiological ligands. These include endogenous (Ca^{2+}, ATP, and Mg^{2+}) and exogenous (caffeine, halothane, ryanodine, dantrolene, and 4-Chloro-*m*-cresol (4-CmC)) ligands. The Ca^{2+}-release channel is activated by μM Ca^{2+}, mM ATP, nM ryanodine, caffeine, 4-CmC (a preservative added to some intravenous medications (Herrmann-Frank *et al.*, 1996)), halothane and alkaline pH. Inhibitors of the channel are mM Ca^{2+}, mM Mg^{2+}, μM ryanodine, dantrolene and acidic pH (Loke and MacLennan, 1998; Palnitkar *et al.*, 1997; Samso and Wagenknecht, 1998;

Valdivia *et al.*, 1991; Zorzato *et al.*, 1990). According to Chen *et al* (1998) Ca^{2+} is the essential regulator of the ryanodine receptor and any other modulators exert their effects by influencing the calcium sensitivity of the receptor.

1.2 The Ryanodine Receptor

The ryanodine receptor functions as the Ca^{2+}-release channel in the SR and is the main regulator of calcium concentration in skeletal muscle cells. It is composed of four identical subunits of ~5038 amino acids each with a molecular weight of ~565 kDa (Phillips *et al.*, 1996). At a total molecular weight of ~2260 kDa, the ryanodine receptor is one of the largest proteins and the largest ion channel known. The entire gene is ~160 kb long and contains 106 exons ranging in size from 15 base pairs to 813 base pairs (Phillips *et al.*, 1996).

Three-dimensional studies show that the receptor consists of two main parts – a large cytoplasmic segment comprising ~80% of the structure, and a smaller (~20%) transmembrane domain at the C-terminal end of the protein. Hydropathy studies and sequence analysis have shown that the C-terminal region contains a number of transmembrane domains which are located in the membrane of the sarcoplasmic reticulum (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). The cytoplasmic domain spans the gap between the SR and the transverse tubule (t-tubule) of the muscle cell membrane (Figure 1.2).

There are three known isoforms of the ryanodine receptor – RyR1, RyR2, RyR3 – which are encoded on separate genes and exhibit widespread expression patterns in mammalian tissue (Giannini *et al.*, 1995). RyR1 is expressed mainly in skeletal muscle, but also brain and smooth muscle; RyR2 in cardiac muscle and also brain and endothelial cells; RyR3 in brain, and smooth muscle and epithelial cells (Samso and Wagenknecht, 1998). This widespread expression pattern may also suggest that ryanodine receptors have a role in calcium regulation in a rather large range of tissues (Giannini *et al.*, 1995).

1.3 The Excitation-Contraction Coupling Process

1.3.1 Excitation-Contraction Coupling

Calcium release is caused by depolarisation of the muscle cell membrane. This signal from a neuronal impulse is sensed by the voltage-sensing dihydropyridine receptor (DHPR) in the t-tubule membrane, and passed to the ryanodine receptor in the terminal cisternae of the SR membrane (Figure 1.2). This occurs by a mechanism that is not fully known.

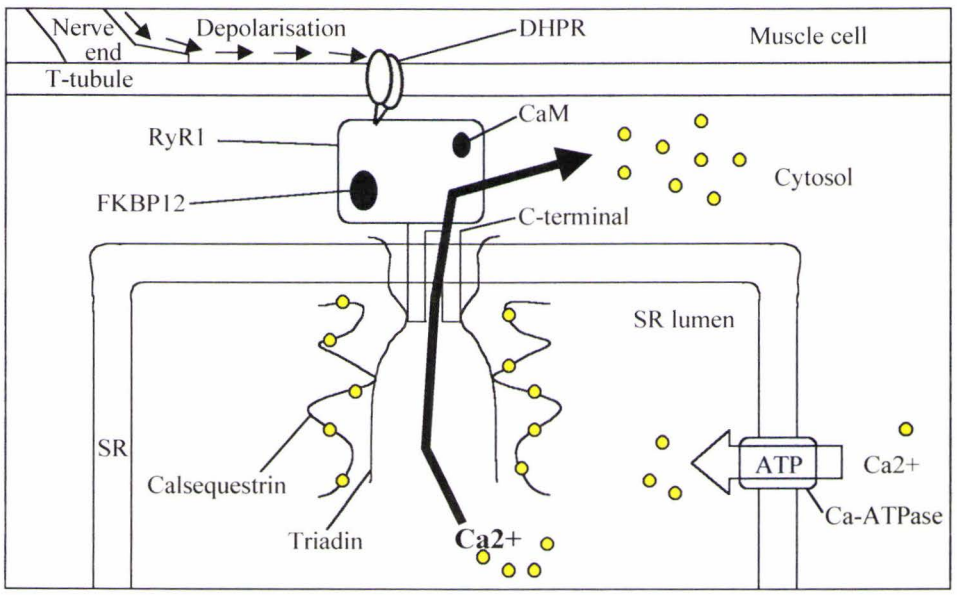

Figure 1.2: The excitation-contraction pathway of calcium release.

A depolarising signal is received by the DHPR and transmitted to the ryanodine receptor causing a release of calcium from the sarcoplasmic reticulum. One subunit of the ryanodine receptor is shown along with the relative position of other proteins that are involved in this process and their relation to the receptor (Adapted from Pessah et al., 1996).

1.3.2 The Ryanodine Receptor and the Dihydropyridine Receptor

The ryanodine receptor is the main component of the Ca^{2+}-release pathway. It is closely associated with the DHPR such that one DHPR associates with every alternate ryanodine receptor structure (Flucher and Franzini-Armstrong, 1996).

The DHPR is a multisubunit complex with each one consisting of four domains. The α1-subunit has a cytoplasmic loop between domain II and III. This has been shown to form a functional interaction with the ryanodine receptor (reviewed in McPherson and Campbell, 1993), but McPherson and Campbell (1993) suggested that there was no direct contact existing between the two proteins. Since then, it has been shown by cross-linking analysis that there is a direct linkage between the ryanodine receptor and the α1-subunit of the DHPR (Murray and Ohlendieck, 1997). Loke and MacLennan (1998) have identified a DHPR binding site near the N-terminal end of the ryanodine receptor.

A more recent study has used NMR spectroscopy to show that the helical structural formation of two peptides, A1 and A2, within the II – III loop of the DHPR is important for interaction with the ryanodine receptor (Casarotto *et al.*, 2000). This group suggest that activation of the ryanodine receptor during excitation-contraction coupling is dependent on the conformational changes within the II – III loop which causes the A1 and A2 peptides to interact strongly with the ryanodine receptor.

There also appear to be other proteins that may interact in some way with the ryanodine receptor and that are involved with the regulation of calcium release. These include triadin and calsequestrin on the luminal side, and FKBP12 and calmodulin (CaM) on the cytoplasmic side of the receptor.

1.3.3 Triadin

Initial investigators thought that triadin bound to both the ryanodine receptor and the DHPR (Caswell *et al.*, 1991). Later analysis indicated that only a short region of triadin was cytoplasmic while the majority of the protein resided in the lumen of the SR (Knudson *et*

al., 1993). This proved that it was highly unlikely to associate with the DHPR although association with the ryanodine receptor was still possible. It was also proposed that triadin serves as an anchor to keep calsequestrin near the ryanodine receptor in the terminal cisternae of the SR (reviewed in McPherson and Campbell, 1993).

Murray and Ohlendieck (1997) demonstrated an association between triadin and the ryanodine receptor, and more recently, ryanodine binding sites have been found at various points on the luminal region of triadin (Caswell *et al.*, 1999). These may bind to a position on the luminal loops in the C-terminal domain of the ryanodine receptor. The precise functional role of triadin in the excitation-contraction coupling process was not well defined in early 1998 (Protasi *et al.*, 1998), although it was suggested that it may be involved in transmitting the Ca^{2+}-release signal to calsequestrin (Pessah *et al.*, 1996).

Later in 1998, another study suggested that triadin was a negative regulator of the ryanodine receptor in that when triadin was present, ^{3}H-ryanodine binding was inhibited and channel opening was decreased (Ohkura *et al.*, 1998). When triadin was removed the ^{3}H-ryanodine binding was increased. These authors suggested that the ryanodine receptor was regulated by triadin in cooperation with calsequestrin in that, while triadin inhibited the channel, calsequestrin was an activator of the channel.

1.3.4 Calsequestrin

Calsequestrin molecules are concentrated in the lumen of the SR. They have a high capacity for binding calcium, which in effect lowers the free calcium concentration within the SR and is one form of storage of calcium in the SR. This binding is not tight and signals can cause release of calcium in response to opening of the ryanodine receptor channel (Murray and Ohlendieck, 1997; Pessah *et al.*, 1996; Szegedi *et al.*, 1999). Murray and Ohlendieck (1997) showed in their linkage studies, that although calsequestrin was present in the terminal cisternae, it was not found in a complex of the ryanodine receptor and the DHPR.

Later in the same year, it was shown that calsequestrin could form a complex with the ryanodine receptor and also the DHPR and triadin (Zhang *et al.*, 1997). This group found that junctin, another membrane protein, played a part in stabilising the complex and anchoring calsequestrin to the ryanodine receptor. More recently it has been found that when microsomal preparations are treated with halothane, a complex forms that contains the ryanodine receptor, DHPR as well as calsequestrin (Froemming *et al.*, 1999). Although these authors suggested that calsequestrin does complex with the ryanodine receptor, it is still unknown whether it is a direct or indirect association.

Szegedi *et al* (1999) have recently discovered that calsequestrin, when dephosphorylated, can regulate the ryanodine receptor and stimulate the subsequent release of calcium. In addition Herzog *et al* (2000) suggested that both phosphorylated and dephosphorylated calsequestrin could bind to the ryanodine receptor. This could prove to be a mechanism by which calcium is released from the SR via the alternate ryanodine receptors that are not associated with the voltage-sensing DHPRs. The signal or mechanism of phosphorylation and dephosphorylation is not known. Therefore, it remains to be seen whether it is still a depolarisation signal that directs the change via some other intermediate.

1.3.5 FKBP12

FKBP12 is a 12 kDa protein of the immunophilin family that binds the immunosuppressant drug FK-506 (Jayaraman *et al.*, 1992). This protein has been identified as part of the three-dimensional structure of the ryanodine receptor. One FKBP12 molecule has been found to associate with each of the cytoplasmic subunits of the ryanodine receptor and modulate its activity by stabilising the closed conformation of the receptor (Samso and Wagenknecht, 1998).

Although FKBP12 may associate tightly with the ryanodine receptor and modulate channel function, it can be exchanged with a soluble form or an isoform, or removed by drug treatment (Qi *et al.*, 1998) with subsequent changes in channel function. The initial tight binding of FKBP12 to the ryanodine receptor was discovered to be a weaker interaction as FKBP12 could be dissociated with CHAPS detergent (Ogawa *et al.*, 1999). Its close

association with the ryanodine receptor indicates that it has a role in the excitation-contraction coupling process, which was confirmed in the study by Qi *et al* (1998).

1.3.6 Calmodulin

Calmodulin is the other protein that interacts with, and regulates the ryanodine receptor in a calcium dependent manner. CaM inhibits the channel in the absence of calcium or in the presence of higher than μM calcium. (Ogawa *et al.*, 1999). When the channel has been activated by μM calcium, CaM will bind to the ryanodine receptor and inhibits the opening of the channel (Samso and Wagenknecht, 1998). When the intracellular concentration is in the nM range, more CaM binds and the receptor is activated. CaM binding sites on the ryanodine receptor have been identified in the central region (O'Driscoll *et al.*, 1996) and also in the C-terminal domain (Takeshima *et al.*, 1989).

1.4 Important Regions on the Ryanodine Receptor

1.4.1 Regulatory Regions

Calcium release and ryanodine binding are modulated by various ligands – Ca^{2+}, Mg^{2+}, ATP, CaM and ryanodine. Binding sites for these modulators have been predicted from primary sequence analysis and binding studies, and have been localised to three main regions on the receptor: the N-terminal domain, the centre and the C-terminal domain (Figure 1.3).

The conformation of the ryanodine receptor, mediated by the N-terminal region, has been shown to be important for ryanodine binding. When portions of the skeletal muscle N-terminal domain were replaced by the identical cardiac isoform segments of the ryanodine receptor, the high affinity binding site was lost (Nakai *et al.*, 1999). This is because the skeletal muscle and cardiac muscle ryanodine receptors behave differently in their ryanodine binding properties.

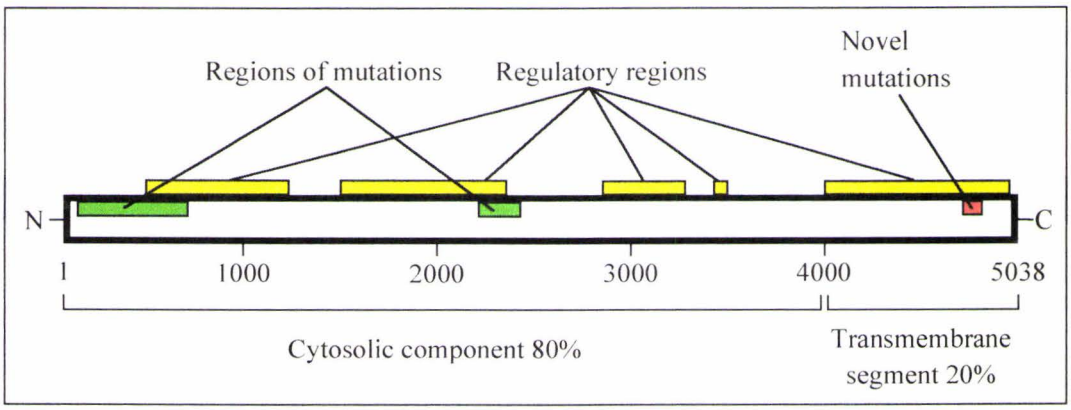

Figure 1.3: A linear representation of the location of the regulatory and mutation sites on the ryanodine receptor.

Regulatory regions are found over widespread areas of the receptor. The mutations are clustered in more compact regions. The transmembrane region of the C-terminal domain is almost entirely composed of regulator binding sites and contains the recently identified novel mutations.

Sites on the N-terminal region include a high affinity ryanodine binding site between amino acid residues 500 and 1300 (Chen *et al.*, 1993b). ATP binds between amino acids 1194 and 1199 (Zorzato *et al.*, 1990) and calcium binds between residues 1641 and 2437 (Samso and Wagenknecht, 1998).

The central region contains two main blocks of modulator binding sites. A low affinity binding site for calcium exists between amino acid residues 1872 and 1923 (Zorzato *et al.*, 1990). Calmodulin binds at several sites including between residues 2807 – 2840, 2909 – 2930, 2937 – 3225 and 3614 – 3637 (O'Driscoll *et al.*, 1996; Takeshima *et al.*, 1989; Zorzato *et al.*, 1990).

The C-terminal region contains binding sites for ATP, Ca^{2+}, CaM and ryanodine. ATP binds at residues 4447 – 4457 (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). Calcium binds between amino acids 4253 – 4264, 4407 – 4416 and 4489 – 4499 (Chen *et al.*, 1992;

Takeshima *et al.*, 1989). CaM binds between residues 4295 and 4325 (Takeshima *et al.*, 1989). Ryanodine has a low and high affinity binding site between residue 4475 and the end of the C-terminal domain (Callaway *et al.*, 1994).

No binding sites have been found for Mg^{2+} (Zorzato *et al.*, 1990).

The C-terminal domain has also been found to have a major role in calcium activation and inactivation in skeletal muscle ryanodine receptors (Nakai *et al.*, 1999). When the C-terminal domain of the skeletal muscle receptor was exchanged with the same part in the cardiac muscle receptor, the channel inactivation was decreased at high calcium concentrations. Another group has also shown that the C-terminal domain contains low affinity calcium binding sites (Du and MacLennan, 1999) which have an effect on inactivation of the channel. Cardiac muscle receptors are not inactivated by mM calcium like skeletal muscle receptors, but both are activated by μM calcium (Du and MacLennan, 1999), therefore the skeletal muscle sequence can be exchanged for cardiac receptor sequence as was done in these two studies to examine the effects of calcium activation and inactivation.

1.4.2 Location of Mutations associated with Malignant Hyperthermia

The known mutations are located in clusters near or within the identified regulatory regions. There are two main sites: one in the N-terminal domain (residues 35 – 614) and one in the central portion of the receptor (residues 2163 – 2458) (Figure 1.3).

A mutation in one of the modulator binding sequences would be expected to cause an alteration or reduction in the binding of that modulator leading to an alteration in the calcium release or ryanodine binding properties of the receptor. This occurs in malignant hyperthermia susceptible (MHS) muscle – calcium release and ryanodine binding is greatly enhanced. Seventeen mutations in the N-terminal domain and central region have been biochemically characterised and they have been shown to be more sensitive to caffeine and halothane (the *in vitro* diagnostic indicators) by exhibiting an increase in calcium release

and/or ryanodine binding (Censier *et al.*, 1998; Richter *et al.*, 1997; Tong *et al.*, 1997). A recent study suggests that there is an interaction between the N-terminal and central domains, where most mutations are clustered (Yamamoto *et al.*, 2000). In wild type channels an interaction occurs which closes the channel and regulates it accordingly. When a mutation exists, the interaction between the two domains prevents closure of the channel so that it remains open and increases the sensitivity of the channel to various modulators. This would account for the great exit of calcium ions during an MH crisis.

Mutations which have been identified include: C35R, R163C, G248R, G341R, I403M, Y522S, R552W, R614C, R614L, R2163C, R2163H, V2168M, T2206M, T2206R, G2434R, R2435L, R2435H (R2436H), R2454C, R2454H, R2458C and R2458H (Jurkat-Rott *et al.*, 2000; McCarthy *et al.*, 2000). Another mutation has recently been identified, R2452W, which occurs in the central region of the receptor (Chamley *et al.*, 2000)

Two mutations have recently been identified in a different region of the receptor. They are located in the C-terminal domain near the third main regulatory site T4826I and H4833Y (Brown *et al.*, 2000; Stowell *et al.*, 1999). A third mutation has also been identified in this region, I4898T, but it is linked to another myopathy called central core disease (CDD), and not to MH (Lynch *et al.*, 1999). This brings the total number of MH- or CCD-associated mutations to 25.

1.5 The C-terminal Region

1.5.1 Models of Transmembrane Domains

The C-terminal domain is an important region of the ryanodine receptor in that it contains a large regulatory region in which three MH- or CCD-associated mutations have been identified, two of which have not been characterised. It is also the region that contains the transmembrane (TM) domains.

Sequence analysis has revealed the presence of four (Takeshima *et al.*, 1989) or ten (Zorzato *et al.*, 1990) TM domains in the C-terminal fifth of the receptor. There are still

discrepancies with the accuracy of the models with some groups supporting either one or the other. Antibodies to selected regions were used to identify luminal sites in the C-terminal region (Grunwald and Meissner, 1995) which supported the 4-TM model. Balshaw *et al* (1999) also reviewed that the 4-TM model is favoured based on some studies with tryptic digestion and deletion mutations. Other research groups deduced that their studies of the three-dimensional structure of the receptor support the 10-TM region model (reviewed in Samso and Wagenknecht, 1998). Chen *et al* (1993b) also support this model after their tryptic digestion studies of ryanodine receptors in SR membrane vesicles which revealed that three fragments associate with the membrane. Four TM regions would be found in only one fragment whereas all three fragments would be needed to support the 10-TM model.

Other support for the 10-TM model has arisen from the study of the effects of mutations in a highly conserved hydrophobic region corresponding to the TM-9 domain in the Zorzato *et al* (1990) model (Du *et al.*, 1998a; Zhao *et al.*, 1999). Bhat *et al* (1997) found that the entire C-terminal domain (~20% of the receptor) could form a fully functional channel, which would suggest that the 10-TM model is supported as all the 10 transmembrane domains were in the segment they studied. On the other hand, a shortened version of the ryanodine receptor was isolated in brain tissue (Takeshima *et al.*, 1993), and was highly homologous to the last ~2.4 kb of the C-terminal end in skeletal muscle containing the four transmembrane domains of the Takeshima *et al* (1989) model. However, this shortened C-terminal region did not function as a full-length channel would.

In the Zorzato et *al* (1990) model, the TM domains are found between amino acids 3982 – 4003, 4021 – 4040, 4277 – 4301, 4342 – 4362, 4559 – 4581, 4648 – 4672, 4789 – 4821, 4837 – 4857, 4879 – 4899 and 4914 – 4938. The Takeshima *et al* (1989) model places the domains between residues 4564 – 4581, 4640 – 4665, 4835 – 4860 and 4917 – 4937.

Although it is still unclear whether the 4-TM model is more accurate than the ten, both models support an even number of TM domains. This is consistent with the overall

structure of the ryanodine receptor which has an ~80% cytoplasmic N-terminal portion and a small cytoplasmic C-terminal tail with the TM region in between.

1.5.2 The Calcium Channel

Initially it was assumed from sequence analysis that the TM segments formed the membrane-bound region of the calcium channel. It was also found that cDNA encoding the whole ryanodine receptor could be expressed in cells to form a functionally active calcium release channel (Chen *et al.*, 1993a).

Earlier in the same year, Takeshima *et al* (1993) identified a 2.4 kb mRNA species in brain tissue that hybridised to the C-terminal 656 amino acid region of the ryanodine receptor. The brain does express an isoform of the full-length receptor but this shortened form appeared to be derived from the C-terminal domain of the skeletal muscle ryanodine receptor using Met^{4382} as the initiation codon. Expression studies showed that this shortened form could produce a membrane protein with only four TM regions, but they could not obtain measurable ryanodine binding or calcium release results comparable with full-length ryanodine receptors.

More recent studies (Bhat *et al.*, 1997) have shown that the C-terminal domain alone is sufficient for channel activity. Expression of the last 1377 residues of the ryanodine receptor in CHO cells could produce a functionally active calcium release channel, which showed similar properties to the full-length version. The C-terminal domain contains calcium and ryanodine binding sites (Callaway *et al.*, 1994; Chen *et al.*, 1992). The main difference with the truncated version was an inability to be inactivated by calcium, suggesting that the calcium binding sites in the cytoplasmic domain have a different function than sites in the C-terminal domain.

A highly conserved region identified in the C-terminal domain of all ryanodine receptors can reduce or totally abolish ^{3}H-ryanodine binding depending on the site of the point mutation within the region (Zhao *et al.*, 1999). This region relates to amino acids 4891 – 4900 in human skeletal muscle ryanodine receptor (refer to appendix 5), and this group

suggests that it is probably the pore forming segment corresponding to the TM-9 domain in the Zorzato *et al* (1990) model. This is the location of the CCD mutation I4898T (Lynch *et al*., 1999) which abolishes ryanodine binding.

1.5.3 Mutations

Lynch *et al* (1999) have identified a mutation, I4898T, in the C-terminal domain of the ryanodine receptor in a large family that are all affected by a severe form of central core disease. CCD is also an autosomal, dominantly inherited disorder characterised by hypotonia and muscle weakness that presents early in life. It is closely associated with MH in that the same gene is involved and five mutations that are linked to MH are also linked to CCD (Tong *et al*., 1997). Both conditions result from an abnormality in the calcium release mechanism in skeletal muscle.

The ryanodine receptor with the I4898T mutation appears to be in a closed state as ryanodine binding was greatly reduced. The maximum Ca^{2+}-release level was greatly decreased and the intracellular concentration of calcium in resting cells was significantly increased from normal. This suggests that the mutant channel may be 'leaky' as calcium stores in the SR were also greatly reduced in resting muscle cells (Lynch *et al*., 1999).

The I4898T mutation appeared to behave differently to other mutations in the ryanodine receptor in that it reduced ryanodine binding and seemed to inactivate the channel (Balshaw *et al*., 1999). The ryanodine receptor with this mutation also had almost no response to caffeine or halothane, which strongly activate channel activity in MHS muscle. Zhao *et al* (1999) have also confirmed the effect of the I4898T mutation which is located in the TM-9 highly conserved region of the ryanodine receptor.

The Takeshima *et al* (1989) model places the I4898T mutation in a luminal loop of the receptor whereas in the Zorzato *et al* (1990) model it is the second to last amino acid in the ninth transmembrane domain heading into the luminal side (see Figure 1.4). Grunwald and Meissner's (1995) antibody studies also suggest that it is found in a luminal loop. The mutation has its greatest effect on calcium sensitivity (Lynch *et al*., 1999), therefore it may

disrupt a luminal calcium binding site, or prevent triadin binding which prevents anchoring of the calsequestrin/Ca^{2+} complexes at the terminal cisternae of the SR.

The observation of low calcium stores in the lumen of the SR and high resting calcium concentration in the cytosol is consistent with this family having no MH episodes on exposure to anaesthetic agents (Lynch *et al.*, 1999). This may mean that this particular mutation is the first to be discovered on the ryanodine receptor that is solely responsible for CCD and not MH.

In light of this, and because other mutations in the N-terminal and central domains altered caffeine and halothane sensitivity, a study was carried out to determine the effects of the C-terminal domain mutations on the ryanodine receptor (Du *et al.*, 2000). This group replaced amino acids 4187 – 4628, the most divergent region in the C-terminal domain, with cardiac muscle receptor sequence and observed an altered sensitivity of the channel to caffeine and calcium. This would suggest that mutations in this, and other regions of the C-terminal domain could have a predictable effect on the ryanodine receptor.

The other two mutations identified in the C-terminal domain, T4826I and H4833Y (Brown *et al.*, 2000; Stowell *et al.*, 1999), occur in families that are susceptible to MH. There is no evidence of CCD in either of these families.

Biochemical analysis of these mutations has yet to be carried out to determine the effects of ryanodine binding and calcium release. It would be assumed that as they are both from MH families, as opposed to the Lynch *et al* (1999) CCD family, that both ryanodine binding and calcium release would be increased.

These two mutations are located on different sides of the SR membrane depending on which model is employed (Figure 1.4). The Takeshima *et al* (1989) model places the location of the mutations on the cytosolic side whereas in the Zorzato *et al* (1990) model the mutations would be on the luminal side of the SR. This is in contrast to the I4898T mutation. Regulation of the channel with either of these mutations would be expected to be

altered. But until the structure of the transmembrane domains is confirmed, the precise molecular effects of modulators on channel activity can not be defined. Some modulators may have more of an effect than others depending on which side of the SR membrane the mutation is on in relation to the TM model and binding site positions. Specific antibody binding studies could confirm the cellular location of the mutation and provide a clearer view as to which TM model is more accurate.

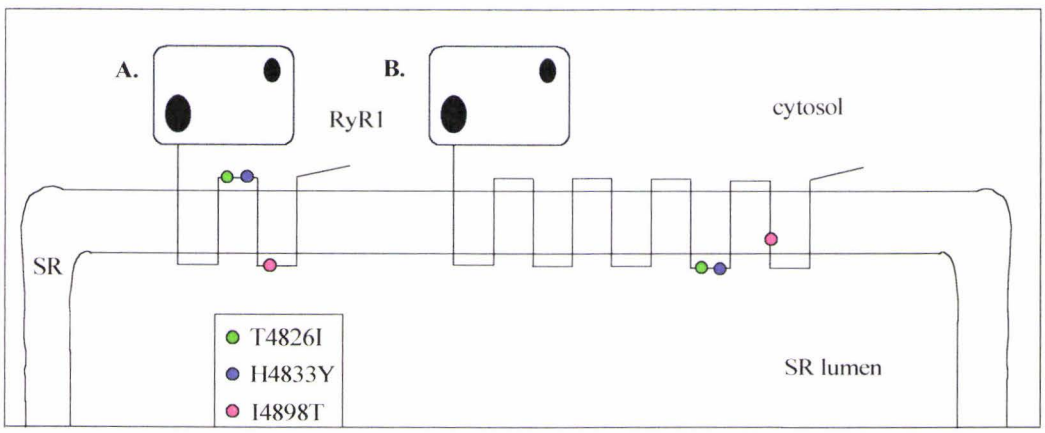

Figure 1.4: Schematic diagram of the location of the C-terminal domain mutations in relation to the transmembrane domain models.

(A) The Takeshima et al (1989) 4-transmembrane domain model places the T4826I and the H4833Y mutations on the cytosolic side of the SR membrane and the I4898T mutation on the luminal side of the SR membrane. (B) The Zorzato et al (1990) 10-transmembrane domain model places the T4826I and the H4833Y mutations on the luminal side of the SR membrane and the I4898T mutation at the second to last position on the ninth transmembrane domain, closest to the luminal side of the SR membrane.

1.6 Significance of this Project

A total of 25 published mutations have now been identified in the ryanodine receptor of MHS individuals. Most of these occur within one of two groups in either the N-terminal

domain or the central region of the receptor. Eighteen of these mutations have been biochemically characterised and have been shown to be causative of the abnormal calcium release observed in MH muscle. Sixteen have been linked to only MH in humans, six have been linked to both MH and CCD (Jurkat-Rott *et al.*, 2000; McCarthy *et al.*, 2000; Tong *et al.*, 1997) and one has been linked to CCD but not MH (Lynch *et al.*, 1999).

Three mutations are located in the regulatory region within the C-terminal domain. One of these mutations appears to be linked only to CCD and exhibits reduced calcium release and ryanodine binding, indicative of an inactive or closed channel. The other two mutations have not yet been biochemically characterised although they are associated with MH.

1.7 Aims of the Project

This research project focuses on the C-terminal domain that includes a large regulatory region containing these novel mutations. The overall objective was to biochemically characterise the T4826I and H4833Y mutations in relation to their calcium release and ryanodine binding properties.

Sarcoplasmic reticulum vesicles containing ryanodine receptors from normal skeletal muscle samples were isolated and the presence of receptors was determined by ^{3}H-ryanodine binding or by immunoblotting.

The C-terminal domain of the ryanodine receptor was constructed by RT-PCR and expression studies were started. This would enable the introduction of point mutations into the C-terminal domain regulatory region so that the calcium release and ryanodine binding effects could be studied using recombinant protein.